AMENDMENTS TO THE SPECIFICATION

Please amend the paragraph at page 7, line 8 as follows:

New adjuvants, such as the Ribi Adjuvant System (RAS) have been designed to substitute highly purified bacterial components for *M. tuberculosis* in order to maintain the immune stimulatory properties of CFA without the side effects. A variation of RAS, Detox™ adjuvant, is currently in clinical trials as a component of cancer vaccines (NCI-V98-1489, NCI-96-C-0139). Others, such as Hunter's ~~TiterMax~~ TITERMAX™, which is has not been approved for clinical use but has been extensively characterized in animal systems, use completely synthetic compounds.

Please amend the paragraph at page 7, line 23 as follows:

Korbelik's group reported results using immuno-adjuvant PDT in 1993 (Korbelik *et al.*, 1993). Initially, the group administered the immunostimulant schizophyllan (SPG), a glucan derived from *Schizophyllum communae*, in a series of intramuscular injections into the hind leg of mice bearing a squamous cell carcinoma solid tumor grown intradermally over the sacral region of the back. ~~Photofrin~~ PHOTOFRIN®-based PDT was administered either 48 hours after the last SPG treatment or 24 hours before the first SPG injection. SPG therapy before PDT enhanced the effect of PDT on tumor cure whereas immunotherapy after PDT had no effect (Krosl and Korbelik, 1994).

Please amend the paragraph at page 8, line 6 as follows:

Another study found that administering the macrophage activating factor vitamin D_3 binding protein macrophage activating factor (DBPMAF) intraperitoneally and peritumorally in a series starting immediately following ~~Photofrin~~PHOTOFRIN®-sensitized PDT enhanced the PDT effect on tumor cures (Korbelik *et al.*, 1997). Later, the group examined the use of BCG and a purified and deproteinized preparation of the mycobacterium cell wall extract (MCWE) that is distributed by Bioniche Inc. (London, Ont. Can.) as ~~Regressin~~ REGRESSIN®, combined with PDT sensitized with ~~Photofrin~~ PHOTOFRIN®, ~~Verteporfin~~ verteporfin, zinc(II)-phthalocyanine (ZnPC), and *meta*tetrahydroxyphenyl-chlorin (mThPC). A single injection of either MCWE or BCG directly

beneath the tumor mass and immediately following PDT resulted in enhanced tumor cure rates (Korbelik and Cecic, 1998).

Please amend the paragraph at page 28, line 14 as follows:

Male, C57BL/6 mice were obtained from Charles River Canada (Montreal, QC) at 6 to 8 weeks of age. The B16-F0 and B16-F1 melanoma cell lines were obtained from the American Type Tissue Collection (ATCC™) (Manassas, Virginia) and grown as cell cultures in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (Sigma). The cells adhered to tissue culture plates, were removed for passage with 0.25% trypsin with 1.0 mM ethylenediaminetetraacetic acid (EDTA) (Gibco), and were cryo-preserved in liquid nitrogen in DMEM plus 40% FBS and 10% DMSO. Mice were injected with 5×10^5 tumor cells in a total volume of 50 μL subcutaneously into the shaved, right flank. The tumor size was monitored daily by measuring the diameter with vernier calipers and were treated when the tumors reached approximately 5 mm in diameter. In initial experiments, the B16-F0 and B16-F1 were characterized with respect to *in vivo* growth rates and metastatic potential and were found to be identical. Subsequently the B16-F1 cell line was used for all experiments.

Please amend the paragraph at page 29, line 3 as follows:

PDT treatment of mice bearing the B16-F1 tumor was performed as previously described for the M1 rhabdomyosarcoma mouse tumor (Richter *et al.*, 1987; Richter *et al.*, 1988; Richter *et al.*, 1991). Each mouse was weighed, warmed under infrared light for less than 5 min to dilate the blood vessels, restrained, and injected intravenously (tail vein) with ~~Verteporfin~~ verteporfin at a concentration of 1.0 mg/kg body weight using a 28G needle. Thirty minutes later, animals were restrained and half of the animals were injected intratumorally with 50 μL of ~~Titermax~~ TITERMAX™ adjuvant (Sigma) prepared as an emulsion with sterile phosphate buffered saline (PBS) according to the manufacturers specifications. Animals were then exposed to a light dose of 100 J/cm^2 in a circular area encompassing the tumor of 1 cm diameter at 688 nm wavelength. The power density was 70 mW/cm^2 and resulted in treatment times of 24 min per animal. Following treatment, animals were monitored daily for tumor response.

Please amend the paragraph at page 31, line 11 as follows:

Seven to 10 days following therapy, animals are sacrificed and inguinal, axillary, cervical, and periaortic lymph nodes are aseptically removed. A single cell suspension is produced from the lymph nodes and this is cultured in half-area, 96-well tissue culture plates (Corning) in the presence of titrations of freeze/thawed tumour cells and irradiated syngeneic splenocytes depleted of erythrocytes as accessory cells. The cells are cultured in the presence of recombinant interleukin-2 (Sigma), and concanavalin A (ConA) (Sigma) is utilized as a positive control to assess the proliferative capacity of lymphocytes. Following 3 to 5 days of culture, the degree of proliferation is assessed using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (Owen's reagent, MTS, from ~~Promega~~ PROMEGA™), a variation of the MTT assay which produces a soluble formazan product which absorbs light at 490 nm. The degree of proliferation is calculated by comparing the means of at least triplicate test wells to the means of lymphocytes cultured without antigen or mitogen (test mean - MTS background ÷ control mean - MTS background x 100 = percent proliferation).

Please amend the paragraph at page 31, lines 25 as follows:

The assays may be performed using the commercial, experimental adjuvant, Ribi Adjuvant System (RAS) (Corixa) or ~~Detox~~ DETOX™ B-SE (Corixa) and alum for comparison.

Please amend the paragraph at page 32, line 11 as follows:

Liposomal ~~verteporfin~~ verteporfin is injected at a dosage of ~~14 mg/m2~~ 14 mg/m^2 of body surface area, which is a higher dose than for treating AMD. One to three hours later, diode laser light is applied at a rate of approximately ~~200mW/cm2~~ 200mW/cm^2 for a total dosage of ~~120-180J/cm2~~ 120-180J/cm^2 to the lesion being treated. The dosage of the ~~Detox~~ DETOX™ adjuvant, which is injected into the lesion after PDT, provides in the range of 100-200μg of the cell wall

skeleton component, and 20-30μg of the monophosphoryl lipid A component. This procedure is carried out at approximately 2 week intervals. Preferably there are 3 treatments.

Please amend the paragraph at page 47, line 13 as follows:

Solubilizers such as ~~Cremophor~~ Cremophor™ types, preferably ~~Cremophor~~ Cremophor™ RH 40, or Tween types or other customary solubilisers, may be added to the solutions of the invention in standard amounts.

Please amend the paragraph at page 53, line 14 as follows:

Male, C57BL/6 mice were obtained from Charles River Canada (Montreal, QC) at 6 to 8 weeks of age. The B16-F0 and B16-F1 melanoma cell lines were obtained from the American Type Tissue Collection (ATCC™) (Manassas, Virginia) and grown as cell cultures in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (Sigma). The cells adhered to tissue culture plates, were removed for passage with 0.25% trypsin with 1.0 mM ethylenediaminetetraacetic acid (EDTA) (Gibco), and were cryo-preserved in liquid nitrogen in DMEM plus 40% FBS and 10% DMSO. Mice were injected with 5×10^5 tumor cells in a total volume of 50 μL subcutaneously into the shaved, right flank. The tumor size was monitored daily by measuring the diameter with vernier calipers and were treated when the tumors reached approximately 5 mm in diameter. In initial experiments, the B16-F0 and B16-F1 were characterized with respect to *in vivo* growth rates and metastatic potential and were found to be identical. Subsequently the B16-F1 cell line was used for all experiments. The injected tumor cells do not result in a representative model of tumor metastasis.

Please amend the paragraph at page 54, line 4 as follows:

PDT treatment of mice bearing the B16-F1 tumor was performed as previously described for the M1 rhabdomyosarcoma mouse tumor (Richter *et al.*, 1987; Richter *et al.*, 1988; Richter *et al.*, 1991). Each mouse was weighed, warmed under infrared light for less than 5 min to dilate the blood

vessels, restrained, and injected intravenously (tail vein) with ~~Verteporfin~~ verteporfin at a concentration of 1.0 mg/kg body weight using a 28G needle. Thirty minutes later, animals were restrained and half of the animals were injected intratumorally with 50 μL of ~~Titermax~~ TITERMAX™ adjuvant (Sigma) prepared as an emulsion with sterile phosphate buffered saline (PBS) according to the manufacturers specifications. Animals were then exposed to a light dose of 100 J/cm^2 in a circular area encompassing the tumor of 1 cm diameter at 688 nm wavelength. The power density was 70 mW/cm^2 and resulted in treatment times of 24 min per animal. Following treatment, animals were monitored daily for tumor response.

Please amend the paragraph at page 57, line 18 as follows:

Mice used in the experiments described below were implanted intradermally with a suspension of 2 x 10^4 M1 (rhabdomyosarcoma) cells on the right flank. Approximately six to ten days after implantation, the site was shaved and tumor growth measured using vernier calipers. Only mice bearing tumors 4 - 6 mm in diameter were selected for use. Mice treated with PDT or PDV received 1.0 mg/kg of ~~Verteporfin~~ verteporfin for Injection (VFI) administered intravenously by tail vein injection. Immediately after injection, the mice were placed in a darkened row of an enclosed, ventilated animal rack (condo unit) for 30 minutes. After 30 minutes, each mouse was secured in a metal holder so that the tumor was centered in a 1.0 cm diameter exposure area. The tumor site was exposed to light from an argon pump dye laser for either 14 minutes, to deliver ~~75 J/cm2~~ 75 J/cm^2, or 4 minutes and 40 seconds, to deliver ~~25 J/cm2~~ 25 J/cm^2. The light doses selected were chosen to deliver either an high PDT dose (~~75 J/cm2~~ 75 J/cm^2) or a low PDT dose (~~25 J/cm2~~ 25 J/cm^2). The exposure time in seconds was determined by dividing the light dose (J/cm^2) by light Intensity (W/cm^2).

Please amend the paragraph at page 58, line 3 as follows:

Immediately following illumination and while still immobilized in the metal holder, mice received a single intratumoral injection of 50 μL of the 1:10 dilution ~~Detox~~ DETOX™ B-SE,

designed to deliver the adjuvant to the center of the tumor mass. Mice in treatment Groups 2 and 5 received 50 μL of 1:2.5 dilution following PDT. See Table 1. Mice in treatment Groups 3 and 6 received PDT treatment, but not adjuvant. Mice in Group 7 remained untreated and were monitored for tumor growth only. Mice in treatment Groups 1 and 4 were treated with 50 μL of 1:10 dilution following PDT.

Please amend the paragraph at page 60, line 14 as follows:

Upon reimplantation of tumor into tumor-free mice on Day 20, the tumor-take rate (percentage of re-implanted animals that showed palpable tumor) of mice treated with PDT, with or without immunoadjuvant, was compared with the tumor-take rate in untreated naïve mice. Implantation of naïve mice (Group 7) resulted in 100% tumor take within 10 days (Figure 4A, Table 4). High dose PDT gave approximately 60% fewer tumors (n=9), and this percentage was not changed by the addition of high dose immunoadjuvant (n=5). The lower dose of immunoadjuvant combined with high dose PDT gave more protection against rechallenge (20% take rate, n=6). A similar trend was seen in survival to 30 days post reimplantation, where high dose ~~Detox~~ Detox™-BSE had little or no effect in addition to high dose PDT alone (Figure 4C, Table 5). The lower immunoadjuvant dose gave more prolonged survival.

Please amend the paragraph at page 62, line 3 as follows:

Upon rechallenge of mice treated with lose dose PDT, the higher dose of Detox-BSE gave the greatest degree of protection (0% take rate at 30 days after tumor reimplantation, n=4). The two other treatment groups allowed approximately 30-40% tumor take (Figure 4B, Table 4). The higher dose of ~~Detox~~ Detox™-BSE also gave the longest survival with all reimplanted mice surviving for 30 days past reimplantation (Figure 4D, Table 5). Low dose PDT alone also gave substantial protection relative to controls and was superior to low dose PDT combined with low-dose adjuvant.

Please amend Appendix A as follows:

PARTICULATE ADJUVANTS

-exist as microscopic, insoluble particles
-generally, the immunogen must be incorporated into or associated with the particle.

A. Mineral-based

-insoluble, gel-like precipitate
-mineral formulations are the only adjuvants that are considered safe and effective for use in human vaccines

i. **Aluminum hydroxide (Alhydrogel)**
Superfos chemicals
~~http://www.superfos.com/index.htm~~

a. **SBAS4**
Aluminum salt combined with monophosphoryl lipid A (MPL)
SmithKline Beecham ~~http://www.sb.com/index.html~~

ii. **Aluminum phosphate (Adju-Phos)**
Superfos chemicals
~~http://www.superfos.com/index.htm~~

ii. **Calcium phosphate**
Superfos chemicals
~~http://www.superfos.com/index.htm~~

B. Water-in-oil emulsions

-microdroplets of water, stabilized by surfactant in a continuous oil phase

i. **Freund's Complete Adjuvant (FCA)**

A. a mixture of a non-metabolizable oil (mineral oil), a surfactant (Arlacel A), and mycobacteria (*M. tuberculosis* or *M. butyricum* in Modified FCA)

Superfos chemicals
~~http://www.superfos.com/index.htm~~

ii. **Freund's Incomplete Adjuvant (FIA)**

B. has the same oil/surfactant mixture as FCA but does not contain any mycobacteria

iii. **Montanide Incomplete Seppic Adjuvant (ISA) Adjuvants**
a group of oil/surfactant based adjuvants in which different surfactants are combined with either a non-metabolizable mineral oil, a metabolizable oil, or a mixture of the two. They are prepared for use as an emulsion with aqueous Ag solution. The surfactant for Montanide ISA 50 is mannide oleate, a major component of the surfactant in Freund's adjuvants. The surfactants of the Montanide group undergo strict quality control to guard against contamination by any substances that could cause excessive inflammation, as has been found for some lots of Arlacel A used in Freund's adjuvant. The

various Montanide ISA group of adjuvants are used as water-in-oil emulsions, oil-in-water emulsions, or water-in-oil-in-water emulsions. The different adjuvants accommodate different aqueous phase/oil phase ratios, because of the variety of surfactant and oil combinations. The performance of these adjuvants is said to be similar to Incomplete Freunds Adjuvant for antibody production; however the inflammatory response is usually less.
Seppic, Paris, France

C. C. Oil-in-water emulsions

-microdroplets of squalene or squalane, stabilized with surfactants in a continuous water phase, developed for human clinical trials when combined with immunomodulators

i. **Ribi Adjuvant System (RAS)**
4 components: (1) monophosphoryl lipid A (MPL); (2) trehalose dimycolate (TDM); (3) cell wall skeletons (CWS); (4) *S. typhimurium* mitogen (STM)
Ribi ImmunoChem Research, Inc.
~~http://www.ribi.com/~~

ii. **MF59**
originally developed with N-~~acety~~acetyl-muramyl-L-alanyl-2-(1',2'-dipalmitolyl-sn-glycero-3'-phospho)ethylamide (MTP-PE) however when antibody titer was endpoint, MTP-PE was not required for adjuvant activity
Chiron Corp.
~~http://www.chiron.com/~~

iii. **SBAS4**
combination of ~~monophosphory~~ monophosphoryl lipid A (MPL), QS21, and a proprietary oil in water emulsion
SmithKline Beecham
~~http://www.sb.com/index.html~~

iv. **Detox™**
active ingredients include MPL® (derivative of the lipid A molecule found in gram negative bacteria) and mycobacterial cell wall skeleton
Corixa Corporation
~~http://www.corixa.com~~

v. ~~**Detox B-SE™**~~ **Detox™ B-SE** (Enhanzyn™) for investigational use is supplied in clear glass vials.

Each vial contains: 145 micrograms CWS from M. phlei, 25 micrograms MPL from S. minnesota R595, 8.1 milligrams Squalane F, 0.38 milligrams Polysorbate 80 (USP/NF), 1.62 milligrams Soy Lecithin (NF), and 88 micrograms Sterile Water for Injection (USP)

~~Detox B-SE~~ Detox™ B-SE must be stored refrigerated between 2 and 8°C

D. Immune stimulating complexes (ISCOM)
-open, cage-like structure resulting from the interaction of Quil-A with cholesterol and phosphatidycholine, human clinical trials

E. Liposomes
-single or multilamellar bilayer membrane vesicles comprised of cholesterol and phospholipid
-the immunogen may be membrane-bound or within the intermembrane spaces

F. Nano- and microparticles
-solid particles, biocompatible and biodegradable, synthetic polymers of cyanoacrylates, polycatide coglycolide (PLG) copolymer, antigen must be formulated with particle

NON-PARTICULATE ADJUVANTS

A. Muramyl dipeptide (MDP) and derivatives: Adjuvant peptides

-*N*-acetyl muramyl-L-alanyl-D-isoglutamine is the active component of peptidoglycan extracted from *Mycobacterium*, derivatives are less toxic

i. **threonyl MDP**

ii. **murabutide,*N*-acetylglucosaminyl-MDP (GMDP)**

a. Gerbu Adjuvant
Alternative to FCA. Oil is replaced by water-soluble, aliphatic quaternary amines or bio-degradable esterquats. Mycobacterium is replaced by GMDP.
Gerbu Biotechnik GmbH, Gaiberg, Germany
C-C Biotech
16766 Espola Road
Poway, CA 92064
USA

iii. **murametide**

iv. **nor-MDP**

B. Non-ionic block copolymers
-polymers composed of a region of hydrophobic polyoxypropylene (POP) flanked by regions of hydrophilic polyoxyethylene (POE), not biodegradable

i. ~~TiterMax~~ **TiterMax™**
CytRx Corporation
~~http://www.cytrx.com/~~

iv. **Syntex Adjuvant Formulation-1 (SAF-1)**
Roche Bioscience (formerly Syntex Corp., Palo Alto, CA)
~~http://www.roche.com/pharma/Index.htm~~

iv. **SAF-2**

C. Saponins

-extract of Quillaia saponaria tree, saponin is crude extract of triterpenoids

i. **Quil A**
Partially purified saponin

ii. **Spikoside**
Partially purified saponin

iii. **QS21 (Stimulon)**
Purified, defined entity
Aquila Biopharmaceuticals, Inc. (formerly Cambridge Biotech Corporation)
~~http://www.aquilabio.com/~~

iv. **ISCOPREP™ 703**
Purified, defined entity

D. Lipid A and derivatives

-disaccharide of glucosamine with two phosphate groups and five or six fatty acid chains (C_{12} to C_{16} in length)

i. **monophosphoryl lipid A (MPL)**
removal of the 1' phosphate group from lipid A gives MPL

E. Cytokines

F. Carbohydrate polymers

-polymers of mannose and β1-3 glucose
-proposed as human vaccine adjuvants either mixed with or conjugated with immunogen
-stimulate macrophages and dendritic cells

G. Derivatized polysaccharides

-high molecular weight sulphated dextrans proposed as human vaccine adjuvants

H. Bacterial toxins

-potent mucosal adjuvants in animal models